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Article

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Impact of a (poly)phenol-rich extract from the brown algae *Ascophyllum nodosum* on DNA damage and antioxidant activity in an overweight/obese population

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Running head: Seaweed phenolics, *Ascophyllum nodosum*, inflammation, DNA damage, C-reactive protein (CRP)

Abbreviations used: SPE, seaweed (poly)phenol extract; CRP, C-reactive protein; CVD, cardiovascular disease; ROS, reactive oxygen species; TNF-α, tumour necrocis factor alpha; COX, cyclooxygenase; UUREC, University of Ulster Research Ethics Committee; WISP, Weighed Intake Software Program; TF, tissue factor; TOC, Total oxidative capacity; TMB, tetramethylbenzidine; IL, interleukin; IFN, interferon; TNF, tumour necrosis factor; UHPLC-HRMS: ultra-high performance liquid chromatography-high resolution-mass spectrometry; VIP: variable of importance in projection; OPLS-DA: Orthogonal Partial Least Square Discriminant Analysis.

This trial was registered at clinicaltrials.gov as NCT02295878.

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ABSTRACT

Background: Epidemiological evidence suggests a diet rich in (poly)phenols has beneficial effects on many chronic diseases. A rich source of (poly)phenols can be found in brown seaweed.

Objective: The aim of this study was to investigate the bioavailability and effect of a seaweed (poly)phenol extract from *Ascophyllum nodosum* on DNA damage, oxidative stress, and inflammation *in vivo*.

Design: A randomised double-blind placebo-controlled crossover trial was conducted in 80 participants aged 30-65 years with a BMI $\geq$25kg/m^2. The participants consumed either a 400 mg capsule containing 100 mg of seaweed (poly)phenol and 300 mg maltodextrin or a 400 mg maltodextrin placebo control capsule daily for an 8-week period. Bioactivity was assessed with a panel of blood-based markers including lymphocyte DNA damage, plasma oxidant capacity, C-reactive protein and inflammatory cytokines. To explore the bioavailability of seaweed phenolics, an untargeted metabolomics analysis of urine and plasma samples following seaweed consumption was determined by UHPLC-HR-MS.

Results: Consumption of the seaweed (poly)phenols resulted in a modest decrease DNA damage but only in a subset of the total population who were obese. There were no significant changes in CRP, antioxidant status, inflammatory cytokines or isoprostanes. We identified phlorotannin metabolites including pyrogallol/phloroglucinol-sulfate, hydroxytrifurahol A-glucuronide, dioxinodehydroeckol-glucuronide, diphlorethol sulfates, C-O-C dimers of phloroglucinol sulfate, C-O-C dimers of phloroglucinol and diphlorethol sulfate are considered potential biomarkers of seaweed consumption.

Conclusion: To the best of our knowledge, our work represents the first comprehensive study in human participants investigating the bioactivity and bioavailability of seaweed (poly)phenolics. There was a modest improvement in DNA damage but only in the obese subset

of the total population, and several biomarkers of seaweed consumption, further studies involving stratification according to weight are required to elucidate these findings.

Key words: Seaweed, Phenolic compounds, Inflammation, DNA damage, Oxidative stress, bioavailability

INTRODUCTION

Diets rich in plant-derived foods protect may against chronic degenerative diseases, including cardiovascular disease (CVD), effects attributable in part to highly bioactive (poly)phenolic compounds contained therin (1-3). Fruits and vegetables are a well-known source of (poly)phenols but a less familiar source rich in (poly)phenolic compounds is brown algae which uniquely contains phlorotannins (4) for example .*Ascophyllum nodosum*, a brown alagae common to the British Isles, that is rich in phlorotannin (5). Phlorotannins are oligomers of phloroglucinol whose concentration in seaweed is affected by numerous factors including plant size and age, water salinity, nutrient and heavy metal content, and light intensity changes (6-8).

Phlorotannins, and more commonly brown seaweed extracts, beneficially effect a range of biological processes including modulation of inflammation; reduction of oxidative stress and improvement in cardiovascular function (9-11). However, the evidence base depends heavily upon cell and small animal models with few studies involving humans (12-13). Moreover, species relevance also becomes an issue; in Southeast Asia, *Ecklonia* and *Eisenia* are considered commercially important seaweed species, while from a European perspective *Ascophyllum nodosum* is of interest as it is one of only a few commercially sustainable seaweed species.

Phlorotannin-rich extracts from brown seaweeds have been shown to be effective in controlling inflammation *via* a number of pathways including inhibition of pro-inflammatory cytokines including tumour necrosis factor (TNF)-α and interleukins (IL)-1β and IL-6 (14). Extracts from *Ascophyllum nodosum* and other Fucoid species have shown efficacy in mitigating the effects of oxidative stress by playing an inhibitory role in the generation of reactive oxygen species (ROS); in preventing DNA damage and also in stimulating the production of glutathione in affected cells (15-19). Our initial *in vitro* (15) and acute *in vivo*

(20) studies on the *Ascophyllum nodosum* extract(s) demonstarted antioxidant and anti-inflammatory activity, suggesting that the potential antioxidant and anti-inflammatory benefits from longer term consumption of *Ascophyllum nodosum*-derived (poly)phenols was worthy of further investigation *in vivo.*

A few human and *in vitro* studies have been conducted in relation to the bioactivity of phlorotannin rich extracts from Fucoid species. Clinical studies have been used to evaluate the safe consumption of some of these extracts including a (poly)phenol-enriched extract of the brown seaweeds *Ascophyllum nodosum* and *Fucus vesiculosus* and its effects of on glycaemic response to sucrose (21). However, to the best of our knowledge, the current investigation is the first clinical study aimed to specifically address the effect of a phlorotannin-rich extract from *Ascophyllum nodosum* on oxidative damage to DNA, plasma antioxidant capacity, inflammatory responses and chronic, low level inflammation *in vivo.*

PARTICIPANTS AND METHODS

Seaweed material

Fresh *Ascophyllum nodosum* was supplied by The Hebridean Seaweed Company, Isle of Lewis, Scotland in March 2011. The seaweed biomass was harvested by hand to ensure quality, cleaned of any contaminating sand and fouling organisms and then shipped refrigerated to the processing facility (CEVA) in France where it was immediately chopped and frozen.

Preparation of food-grade seaweed extracts and capsule

A novel (poly)phenol-rich seaweed extract from *Ascophyllum nodosum* was produced by CEVA (France) using food grade solvent based (ethanol:water, 60:40) extraction system that was specifically developed for use with fresh or frozen *Ascophyllum nodosum*. Approximately half of the produced extract was then fractionated using tangential flow ultra-filtration to produce further extracts of varying molecular weight range and with varying (poly)phenol

content. A standardised blended (poly)phenol-rich *Ascophyllum nodosum* extract was formulated by CEVA (**Table 1**) using 175 mg of extract and 50 mg of high molecular weight fraction (>10 kDa cut off) for use in the current study, so as to maximise the seaweed (poly)phenol content (>100 mg per day) avaliable form the extraction of fresh or frozen Ascophyllum nodosum against the need to minimise the level of iodine to within accepted regulatory guidelines (<500 μg per day), potential for heavy metal contamination was also assessed (Table 1). Maltodextrin (175 mg) was added to the capsule formulation as an excipient. Blending was carried out at the food grade CEVA facilities in France. Samples of 400 mg of the *Ascophyllum* (poly)phenol rich blend (SPE) or a placebo which contained 400 mg maltodextrin of and no seaweed (poly)phenols were packed into identical white, opaque, vegetarian capsules by Irish Seaweeds, Belfast, UK and identically sized and match capsules used for the clinical study. The food grade seaweed capsule was characterized by NP-HPLC and LC-MS analysis and has been reported previously (20) (see supplemental figure 2 & 3). Phlorotannins were quantified using the Folin-Ciocalteau Method (22) using phloroglucinol as the standard. In brief, 1 mL of suitability diluted sample was reacted with 1.00 mL of 40 % Folin Ciocalteu reagent for 5 min, and then made alkaline with the addition of 1.00 ml of 100 g/L Na_2CO_3. Absorbance was read at 730 nm after the solution had developed for 1 h at room temperature. Phloroglucinol dihydrate (0-30 mg/L) was used as the standard and was treated in the same way as samples.

Ethics and participants

Ethical approval was received from the Ulster University Research Ethics Committee (UUREC). All participants gave written informed consent. Participants were recruited between May 2011 and August 2011 from Ulster University and the surrounding area. The intervention

study ran between August 2011 and February 2012. The study was registered at clinicaltrials.gov as NCT02295878.

The study was conducted in 80 participants (age range 30-65 years). All participants were apparently healthy, non-smoking, BMI≥25 kg/m^2, omnivores, who did not habitually use vitamin or mineral supplements, as determined using a pre-screening health and lifestyle questionnaire. Pregnant and lactating women, vegetarians and vegans and lactose intolerant individuals were excluded from the study, as were those with chronic medical complications such as diabetes; cardiovascular diseases; autoimmune/inflammatory disorders or those who had chronic medication use including anti-inflammatory agents.

Study design

The study was a 24-week randomised, double-blind, placebo-controlled crossover trial. After obtaining consent, participants were randomly assigned, in blocks of four using a random-number generator (www.randomization.com), to either the intervention or the control. In total, eighty participants were randomised to 2 groups of 40, each starting on either a 400 mg seaweed (poly)phenol extract (SPE) capsule containing 100mg of (poly)phenols or a 400 mg maltodextrin placebo control capsule (Avebe MD14P) daily for an 8-week period. The participants were supplied with all capsules in weekly labelled capsule boxes at the beginning of each phase, which was interspersed by an 8-week washout phase. During the washout phase, the participants were asked to maintain their habitual diet. Participants were asked to bring any unconsumed capsules to their study appointment at the end of each treatment phase. Participants were also contacted weekly by the study researcher to encourage compliance and to discuss any difficulties they were experiencing.

Blood and urine sample collection

Fasting blood samples were collected before and after each phase (week 0, week 8, week 16, and week 24) by venepuncture into EDTA, serum or sodium heparin-containing tubes, as required. All blood samples were processed on ice. Lymphocytes were isolated by using Histopaque-1077, according to the manufacturer's instructions (Sigma Diagnostics, St Louis, MO), and plasma samples were prepared by centrifugation at 1000 x g for 10 min at 4ºC. Serum samples were allowed to clot for 30 min at room temperature and then were centrifuged at 2000 x g for 10 min at 4ºC. Whole blood from sodium-heparin treated tubes was prepared according to the manufacturer's instructions (BD Bioscience Fast Immune Cytokine System) and 24-hour urine samples were prepared by centrifugation at 1000 x g for 10 min at 4ºC. Whole blood, plasma, serum and urine samples were immediately stored at -80ºC, whereas lymphocytes were stored frozen in liquid nitrogen. All biological measurements were carried out at the end of the intervention in batches containing equal numbers of active and control phase samples in each batch, and the researchers were blinded to these samples during analyses. A 24hr urine collection occurred at each time point, volume and pH was measured. The urine sample was mixed and 2x 14ml aliquots removed, centrifuged at 3000rpm for 10 minutes, supernatant was stored -80ºC until use.

Questionnaire assessments

All participants completed a health and lifestyle questionnaire assessing their alcohol intake and physical activity levels, as well as a validated 4-day food diary at the mid-point during each treatment phase (active/placebo) of the study. Data on type of food and corresponding weight was entered into a food analysis database (WISP, Weighed Intake Software Program; Tinuviel Software, Warrington, U.K.) by two independent researchers and the dietary composition calculated.

206

207 **DNA damage in peripheral blood mononuclear cells**

208 Peripheral blood lymphocytes, previously isolated and stored in liquid nitrogen, were
209 thawed and screened for basal single strand breaks (SBs) in DNA using the single cell gel
210 electrophoresis (Comet) assay (23) and adapted by Gill *et al.* (24). Spontaneous DNA SBs are
211 associated with an altered cell function spontaneous DNA SBs are considered appropriate for
212 the substantiation of health claims in the context of protection against generic DNA
213 damage (25). In addition, resistance to induced DNA damage (SB) was measured in
214 lymphocytes subjected to increased oxidative insult *ex vivo* by pre-treating lymphocytes with
215 150µmol H_2O_2/L for 5 min at 4°C, before the measurement of SBs. The mean (percentage
216 DNA in tail) was calculated from 50 cells per gel (each sample in triplicate) and the mean of
217 each set of data were used in the statistical analysis.

218

219 **Plasma total oxidative capacity**

220 Total oxidative capacity (TOC) measures total peroxide levels in plasma, by the reaction of
221 endogenous peroxides with peroxidases, using tetramethylbenzidine (TMB) as the
222 chromogenic substrate (26). The blue colour of TMB turns to yellow after addition of the stop
223 solution and can be measured photometrically at 450 nm. For the assay protocol, 10 µl of
224 standard (freshly-prepared hydrogen peroxide, 0-1 mmol/L) and samples were incubated with
225 200 µl of the reaction mixture consisting of a reaction buffer (phosphate-citrate buffer 0.05M,
226 pH 5.0), TMB solution (1 mg/ml), and peroxidase (>2500U/ml) in a proportion of 100:10:1 in
227 uncoated microtiter plates and incubated at room temperature for 15 min. 50 µl of stop solution
228 ($2MH_2SO_4$) were added into all wells and the absorbance at 450 nm was measured using a
229 microplate reader (GENIOS Tecan). Hydrogen peroxide standard solutions (0-1 mmol/L) for
230 calibration curve were freshly prepared before use.

231

232 **Lipid profile and serum C-reactive protein**

233 Plasma total cholesterol, HDL cholesterol and triglycerides were measured on an Instrument
234 Laboratory (ILAB) 600 (Warrington, UK) autoanalyzer using commercial kits (Roche
235 diagnostics, Lewis, UK) according to kit manufacturer's protocols. Plasma LDL cholesterol
236 was calculated using the Friedewald formula (27).

237 C-reactive protein, an acute phase protein synthesized by the liver in response to
238 inflammatory stimuli, especially the cytokine interleukin (IL)-6, was determined on an ILAB
239 600 autoanalyser using a *quantex* CRP Ultra-Sensitive commercial kit (0.4-18.3 µg/dl) in
240 accordance with manufacturer's instructions.

241

242 **Measurement of inflammatory markers**

243 Intracellular cytokine levels in lymphocyte and monocyte populations and tissue factor (TF)
244 expression were assessed using a whole blood labelling method that utilises flow-cytometry
245 (Fast Immune Cytokine System, BD Biosciences) in accordance with manufacturer's
246 instructions for all participants at all time-points. The method was used to measure intracellular
247 IL-1β, IL-2, IL-6, IL-10, IL-12, interferon (IFN)-γ and tumour necrosis factor (TNF)-α
248 expression in mononuclear cells. Briefly, whole blood was incubated with either
249 lipopolysaccharide or phorbol 12-myristate 13-acetate to activate monocytes and lymphocytes,
250 respectively. Cells were labelled with the appropriate cell surface antibody and cytokine-
251 specific antibody and analysed on a Gallios flow cytometer (Beckman Coulter). The number
252 and percentage of each cell type expressing the cytokine, as well as the mean channel
253 fluorescence was recorded. The cytokine profiles were examined by ratio of TNF-α to IL-10,
254 IL-1β to IL-10, IL-6 to IL-10 and CRP to IL-10, according to Laird et al. (29).

Isoprostanes have been established as chemically stable, highly specific and reliable biomarkers of *in vivo* oxidative stress, and were measured in frozen serum samples using a commercial 8-Isoprostane EIA Kit (Item no. 516351, Cayman Chemicals). This assay is based on the competition between 8-isoprostane and an 8-isoprostane acetylcholinesterase (Ache) conjugate (8-Isoprostane Tracer) for a limited number of 8-isoprostane-specific rabbit antiserum binding sites consequently 8-isoprostane concentrations are measured as a function of turbidity (absorbance).

Processing of urine and plasma.

Urine samples were defrosted, vortexed, centrifuged at 16110 g for 10 min at 5 °C, and passed through 0.45 µm filter discs prior to the analysis of 50 µL aliquots by UHPLC-HR-MS. The extraction of metabolites from the plasma samples has been carried out as described previously (28). Briefly, plasma samples were defrosted, vortexed and 400 µL aliquots were mixed with 10 µL of ascorbic acid (10%, v/v), and 980 µL of 1% formic acid in acetonitrile. One µg of rutin was added to the samples as internal standard for plasma extraction efficiency. The samples were then vortexed for 1 min and ultrasonicated for 10 min. After centrifugation at 16,110 g for 15 min, supernatants were reduced to dryness in vacuo using a concentrator plus (Eppendorf, Hamburg, Germany) and resuspended in 150 µL of distilled water containing 1% formic acid and 50 µL of methanol, which was then centrifuged at 16,100 g for 10 min and 10 µL aliquots of the supernatant analysed by UHPLC-HRMS. The recoveries values of the internal standard were of 79 ± 16% (n=78).

Non-targeted analysis of urine and plasma by UHPLC-HR-MS.

Aliquots of selected plasma and urine samples were analysed using a Dionex Ultimate 3000 RS UHPLC system comprising of a UHPLC pump, a PDA detector scanning from 200 to 600

nm, and an autosampler operating at 4 °C (Thermo Scientific). The HPLC conditions were previously described by Corona *et al.,* (20) with some modifications. Briefly, reverse phase separations were carried out using a 100 x 2.1 mm i.d. 1.8 µm Zorbax SB C18 (Agilent) maintained at 25 °C and eluted at a flow rate of 0.2 mL/min with a 50 min gradient of 3-70% of 0.1% acidic methanol in 0.1% aqueous formic acid. After passing through the flow cell of the PDA detector the column eluate was directed to an ExactiveTM Orbitrap mass spectrometer fitted with a heated electrospray ionization probe (Thermo Scientific) operating in negative ionization mode. Analyses were based on scanning from 100 to 1000 m/z, with in-source collision-induced dissociation at 25.0 eV. The capillary temperature was 350 °C, the heater temperature was 150 °C, the sheath gas and the auxillary gas flow rate were both 25 and 5 units, respectively, and the sweep gas was 4 and the spray voltage was 3.00 kv. Data acquisition and processing were carried out using Xcalibur 3.0 software.

Untargeted analysis of the selected urine and plasma samples was performed using mass spectral data from the orbitrap analysis applied to the Compound Discoverer software (version 2, Thermo Fisher Scientific Inc.). The Compound Discover application processes the raw data in called processing workflows that can be defined on the basis of the nature of the experimental setup. In our case, the workflow selected was 'untargeted metabolomics workflow' that includes retention time alignment, component detection, grouping, elemental composition prediction, gap filling, hide chemical background (using blanks), ID using mzCloud and ChemSpider and differential analysis. The parameters were adjusted to our experimental conditions. Samples were grouped and labelled according to our experimental design, either before or after supplementation of seaweed capsule, 4 The output, as peak areas for the detected peaks was used to develop a multivariate data analysis by Orthogonal Partial Least Square Discriminant Analysis (OPLS-DA). Analysis of seaweed phenolics in blood and urine was also undertaken

by HPLC-DAD analysis (reported in Supplemental Tables 4 & 5) in a manner consistent with previous studies (15,20).

Power calculations and statistical analyses

Power calculations were performed for the primary endpoint of the change in DNA damage in peripheral blood mononuclear cells. Based on data from a previous study (24), 72.6 participants were needed to detect a 25% change in DNA damage in lymphocytes (α 0.05).

All values are expressed as mean ± SD, unless otherwise specified. The mean values are reported for all participants (n=78) during both treatment phases (SPE, Maltodextrin). Significant associations (P>0.05) between outcome variables including DNA damage and possible cofounders (age, gender, BMI) were identified at baseline using bivariate correlations or independent t-tests, where appropriate. Therefore, data was also analysed by stratification of increasing risk, including overweight participants (n=42) and obese participants (n=36). All biochemical analysis was conducted in duplicates, unless otherwise stated, and the mean values taken as the final result. For all markers, the results are presented as treatment effects. This was undertaken by calculating individual differences between pre- and post- values for both control and treatment phases for each subject. Paired T-tests were then carried out on the difference scores (post-treatment value *minus* pre-treatment values) for both treatment phases (SPE and Maltodextrin). Significance level was set at P<0.05. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) for Windows version 22.0 (SPSS Inc., Chicago, IL, USA).

Multivariate Data Analysis

Data were obtained as peak areas from the Compound Discover automatic integration software and consisted of 2194 and 3289 potential metabolites (or features) in urine and plasma samples,

respectively. Relative peak areas of the metabolites (normalized by the total urine excretion of each subject) obtained by UPLC-HRMS were imported into MATLAB R2015b (Mathworks, USA). PLS toolbox v.8.5 (Eigenvector, USA) and homemade scripts were used. Principal component analysis (PCA) and hierarchical cluster analysis (HCA) are the most widely used tools to explore similarities and patterns among samples where data grouping are unclear. Moreover, the orthogonal partial least squares discriminate analysis (OPLS-DA) method was performed as a typical supervised multivariate methodology used in metabolomics studies (30,31). Several data pre-processing transformations were performed and evaluated, such as Probabilistic Quote Normalization (PQN), Log transformation, mean centering, pareto scaling and auto-scaling. In our case, PQN and autoscaling were selected as pre-processing techniques to reach the lowest root mean square error (RMSE) in an iterative process. A re-sampling method, cross-validation k-fold cross validation (k=5) was also used to evaluate the number of latent variables (according to the lowest RMSE) and the prediction ability of our models. To reduce the impact of the random split of CV-participants, the mean values of the estimated results were obtained after 20 random 5-fold CV. Urine models provided successful classification results at the cross validation step and achieved good prediction parameters that can be explained by the area under the curve values (1.00 and 0.895). Based on the plasma data set and classes selected a discriminant model could not be developed. Moreover, the Variable Importance in Projection (VIP) is the widely known metric that is used to identify potential markers in metabolomics studies (32). VIP is a weighted sum of squares of the PLS weight which indicates the importance of the variable to the whole model. The cut off VIP value selected in our study was 2. The potential markers were extracted from the two models to compare the results obtained.

RESULTS

354 **Baseline characteristics**

355 Eighty participants (males n=39, females n=41) were enrolled on this 24-week randomised,
356 double-blind, placebo-controlled crossover trial. The intervention was conducted as per the
357 protocol and there were no adverse events associated with the intervention. The study
358 population had a mean group age of 42.7±7.1 years and a mean BMI of 30.2±3.9 kg/m^2. The
359 study had an overall compliance of 97% with 78 participants completing the 24-week study;
360 two participants withdrew from the study at the midpoint for personal reasons and compliance
361 was not significantly different by treatment group or time period (P>0.05) (**Figure 1**). There
362 were no significant differences between the participants in age and physical characteristics at
363 the beginning of either treatment phase (**Table 2**).

364

365 **Habitual dietary intake**

366 Dietary analysis of habitual intake (midpoint) during both treatment phases (SPE,
367 Maltodextrin) of the crossover trial is described in **Table 3**. There were no significant
368 differences between treatment phases for any of the macronutrients or micronutrients analysed
369 indicating that the seaweed phenolic extract did not affect the habitual food consumption
370 patterns in the study population.

371

372 **Effects of seaweed (poly)phenol extract on DNA damage**

373 The basal levels of DNA damage observed in the study were consistent with previous studies
374 with a mean group average of 6.72 ± 2.48% tail DNA (data not shown). In response to an
375 oxidative challenge with 150 µM H_2O_2, DNA damage was increased to an average of
376 approximately 34 ± 7% tail DNA in both the placebo and SPE phase in all participants as
377 reported in **Table 4**. The 8-week intervention with a seaweed phenolic extract resulted in a
378 significant reduction in basal DNA damage, as measured by the Comet assay, in obese

379 participants only (BMI >30 kg/m^2), with a significant reduction (P=0.044) in basal DNA
380 damage observed (**Table 4**). A significant reduction was not observed in the total population
381 (n=78) nor in the overweight participants (n=42), either in terms of a challenge response or in
382 basal levels. Additionally, consumption of seaweed phenolic extract also significantly reduced
383 (P=0.009) basal DNA in males only with a mean group change value of -0.8 ± 2.5 % tail DNA
384 (SPE) compared to 0.9 ±2.8% tail DNA in the control. No significant effects where observed
385 for females.
386
387 **Total oxidative capacity of seaweed phenolics**
388 **Figure 2** shows the total oxidative capacity (peroxide levels) in plasma samples from all
389 participants (n=78), in overweight participants (n=42) and in obese participants (n=36)
390 measured after the placebo and seaweed capsule intervention. There were no significant
391 changes from baseline after either treatment phase (SPE, Maltodextrin) in all participants
392 (n=78) nor in overweight (n=42) or obese (n=36) sub groups. However the consumption of
393 seaweed phenolic extract also significantly reduced (P=0.018) TOC in females only with a
394 mean group change value of -7.44 ± 29.37 (SPE) compared to 4.33 ± 22.36 in the control. No
395 significant effects where observed for males.
396 **Effects of seaweed (poly)phenol extract on blood lipids and CRP**
397 **Table 4** shows the pre- and post- values, as well as the percentage change, for both the
398 placebo phase and the SPE phase for each blood lipid biomarker and CRP. An 8-week
399 supplementation with seaweed (poly)phenol extract did not significantly affect any
400 cardiovascular risk marker.
401 **Effects of seaweed (poly)phenol extract on inflammatory markers**
402 $F_{2\alpha}$-isoprostane levels at baseline were 392 ± 219 pg/ml (data not shown). A high degree of
403 inter-individual variation was observed, and the 8-week SPE intervention did not exhibit a

404 significant effect on this marker of oxidative stress (**Table 4**). There were no significant
405 differences for any of the inflammatory cytokines measured in either treatment phase (SPE,
406 Maltodextrin) for all of the participants (n=78), in the overweight participants (n=42) or in the
407 obese participants (n=36) (**Figure 4**). Nor where significant effects observed in the cytokine
408 profiles when examined by ratio of TNF-α to IL-10, IL-1β to IL-10, IL-6 to IL-10 and CRP to
409 IL-10, according to Laird et al. (29)

410 **Bioavailability of seaweed phenolics, untargeted Analysis**

411 Initially an unsupervised PCA model was carried out (**Figure 4A**) on the urine data from all
412 78 participants. The first component of the PC1 vs PC2 scores plot obtained from the data set
413 explained 23.14% of the total variance showing a clear trend among some of the participants
414 compared to the rest. Moreover, a hierarchical cluster analysis was performed to verify this
415 natural aggrupation pattern among the 78 participants suggesting, two clusters among all the
416 participants (**Figure 4B**) which share, accounting for the inter-individual differences associated
417 with differences in the urinary metabolite profiles. For instance, recent studies suggest that
418 individuals can be clustered into distinct groups based on their gut microbiome composition,
419 functional metabolism (33) or individual responses to obtain markers of a specific treatment,
420 minimizing the inter-individual data that are not the target of the study. In order to elucidate
421 the observed variability between these two groups, two supervised models were performed,
422 one with the urinary profiles from all the participants before the seaweed consumption and
423 another one with the urinary profiles from all the participants after seaweed consumption. Both
424 supervised models verified differences between the two groups of individuals (Group 1 and
425 Group 2) and provided successful classification results at the cross validation step and achieved
426 in the pre-treatment and post-treatment samples 95% and 98% sensitivity (participants of the
427 class of interest correctly assigned to their class) and 100% and 100% specificity (participants
428 not belonging to the class of interest were correctly not assigned to that class), respectively

(**Supplemental Figure 1**). It is noteworthy that even stratifying for BMI category, the multivariate analysis of the urine profiles did not show clear differentiation of the metabolome between obese or overweight individuals.

By using the preliminary information provided by PCA and HCA related to the stratification of the individuals into groups which share a common excretion profile before and after supplementation with seaweed capsules, two data sets were analysed. The individuals were stratified into two groups; Group 1 including 70 participants; and Group 2 including 8 participants (S58, S60, S61, S71, S76, S78, S79, S83, all being overweight individuals). Two supervised OPLS-DA models were built to discriminate according to the seaweed treatment in both models (**Figure 5**). In our study we used two latent variables (LV) and the sensitivity and specificity values were set to 100% at the calibration step recognition ability of the two models. These analyses showed that the human urine metabolome in both groups of participants was modified after the 8-week supplementation with seaweed (poly)phenol extract compared to baseline urine metabolic profiles. Using the loadings plot and the variable importance in projection values (VIP) we ascertained important contributors between the modelled classes and therefore identified the compounds responsible for the difference in the urine metabolic profile before and after 8-weeks seaweed (poly)phenol extract consumption in both groups. VIP is the widely known metric that is used to identify potential markers (metabolites) in metabolomics studies. Further, the urine scores plot allowed the identification of those metabolites which appeared after the seaweed (poly)phenol extract intake (**Figure 5**) in both groups of participants.

The main contributors to metabolome differentiation in the urine before and after seaweed consumption in both groups of participants are described in Supplemental Table 1 and Table 2 (Supplemental Material). Positive loading values (x axis) and higher VIP values (VIP>2.0)

were detected in urine after seaweed consumption and were the responsible of the observed differences between the participants classes (post ingestion vs non ingestion of seaweed (poly)phenol extract). Urine metabolites tentatively identified as pyrogallol/phloroglucinol sulfate, hydroxytrifurahol A-sulfate and dioxinodehydroeckol glucuronide are considered clear biomarkers of seaweed consumption in Group 1 while C-O-C dimer of phloroglucinol-sulfate, C-O-C-dimer of phloroglucinol, fucophloroethol-glucuronide, diphlorethol sulfates and dioxinodehydroeckol glucuronide are those biomarkers of seaweed consumption in Group 2. All of them have shown high values in the variable importance in the projection (values > 2) (**Table 5).**

Based upon the identified seaweed (poly)phenol metabolites, the total amount of seaweed metabolites excreted in urine varied noticeably between participant ranging from 0.001 to 4.140 mmoles, with a group average of 1.29 +/- 0.88 mmoles. 25% of the population (19/78) appeared to be low excretors with urinary seaweed metabolites excretion less than 0.5 mmoles, 55% of the population (43/78) were medium excretors with an urinary seaweed excretion between 0.5 and 2 mmoles, while 20% of the population (16/78) where high excretors with total seaweed metabolites concentrations >2 mmoles (see Supplemental Table 6).

The plasma metabolome demonstrated no clear differentiation for participants in either treatment phase (data not shown), nor with exploration of the data by PCA or HCA. Consequently no exposure biomarkers could be defined for plasma samples taking into account all the participants (n=78).

DISCUSSION

The association of seaweed consumption with reduced risk factors for CVD has been tested largely within *in vitro* or animal models, and only limited human data exists to substantiate the

proposed beneficial properties of seaweeds (12,13,34). To the best of our knowledge this trial is the first comprehensive study to investigate the *in vivo* bioactivity and bioavailability of seaweed (poly)phenolics on biomarkers of inflammation and oxidative stress in overweight and obese individuals. Participant retention rates were high (98%) and self-reported SPE capsule intake (97%) indicated that the intervention was implemented successfully in this group of participants. There was, however, no significant difference between the intervention (SPE capsule) and control (placebo capsule) phases for markers of oxidative stress, antioxidant status or inflammation in the population as a whole (n=78). Subset analysis, stratifying for BMI category, showed a significant albeit modest difference in DNA damage in the obese population (n=36). This is consistent with Park *et al*. (35) who tested astaxanthin, a natural carotenoid in the form of microalgae *Haematococcus pluvialis* in 14 healthy females (2 mg/d extract for 8 weeks) demonstrating a significant reduction in the oxidative damage marker plasma 8-hydroxy-2'-deoxyguanosine. Obese individuals are associated with increased DNA damage, higher oxidant status and increased oxidative damage to macromolecules a group and are at higher risk of chronic disease (36-39). In our study, an overweight/obese population (age 30-65 years and a BMI >25 kg/m^2) was shown to have significantly reduced basal levels of lymphocyte DNA damage (by 23%) but only in the obese population subset (n=36) following supplementation. Both seaweed-based intervention studies support data from *in vitro* studies indicating anti-genotoxic activity for seaweed extracts on a range of cell lines (40,41) including the SPE extract used in the study (15). Although a mechanism was not determined for the observed reduction in DNA damage in the obese group activate Nrf2-mediated cytoprotection may be involved, as seaweed (poly)phenols such as eckol are anti-genotoxic (42) and can activate Nrf2-mediated HO-1 induction (43,44) consistent with effects observed for (poly)phenols from terrestrial sources (45,46). Park *et al*. (35) also observed a significant reduction in plasma CRP levels, an acute marker of inflammation. Although in our study we

observed a 28% decrease in CRP levels in response to seaweed extract consumption, this change was not significant. However, a similar study investigating the effect of consuming *Palmaria palmata* (5 g/day) incorporated into bread found that it significantly increased CRP by 16%, suggesting that *P. palmata* stimulates inflammation rather than reducing it (47). Park *et al*. (35) reported that there was no difference in TNF and IL-2 concentrations, but plasma IFN-γand IL-6 increased on week 8 in participants given 8mg astaxanthin. Within this study, we similarly observed no significant changes in IL-2 or TNF, nor did we see an alteration in IFN-γor IL-6. The seaweed extract tested did not affect immune function (cell mediated and humoral immune responses as tested by the cytokine markers) following supplementation.

In order to evaluate the seaweed molecules responsible for its modest beneficial effects *in vivo* and the potential biological markers linked to seaweed consumption, an untargeted approach comparing the metabolic profiles between urine and plasma samples of 78 individuals who consume seaweed capsules have been carried out. As a first step, we have determined differences in the urine metabolic profiles between participants being able to stratify the individuals into groups which share common excretion metabolite profile before and after seaweed capsule supplementation. Urinary profiles of these two groups were statistically different between each other and the metabolites responsible for this discrimination have been selected as potential biomarkers of seaweed consumption (**Table 5**). Subsequently, the quantification of these potential biomarkers within all participants revealed substantial inter-individual variation in the concentration of seaweed metabolites excreted in urine by the participants ranging from 0.001 to 4.140 mmoles. Besides, the outcomes of the comprehensive multivariate analysis of the metabolite profiles shows, as expected, person-to-person variation in the 0-24h urinary excretion of seaweed (poly)phenols, probably due to differences in gut microbiota and no strict living conditions of the participants. As a consequence of these inter

528 individual differences, the 78 participants involved in this study can be grouped by their
529 dissimilarity to metabolize seaweed (poly)phenols in two well established groups. For instance,
530 group 1 of individuals is characterized by greater excretion of seaweed derived metabolites
531 such as pyrogallol/phloroglucinol sulfate, hydroxytrifurahol A-sulfate and
532 dioxinodehydroeckol; while participants belonging to group 2 C-O-C dimer of phloroglucinol-
533 sulfate, C-O-C dimer of phloroglucinol, fucophloroethol-glucuronide, diphlorethol sulfates and
534 dioxinodehydroeckol glucuronide in low concentrations. This inter-individual variation in the
535 absorption of seaweed polyphenols has been observed in the bioavailability of dietary
536 (poly)phenols such as orange juice, cranberry and pomegranate (48-50).

537 The results from the non-targeted approach confirmed the presence of seaweed phenolic
538 metabolites, and can be divided into a) phase II sulphated and glucuronidated metabolites
539 related to the targeted components described earlier by Corona *et al.* (20) (supplemental Tables
540 4,5); arguably formed in the liver; b) an extended list of unknown compounds which may be
541 potential breakdown products or metabolites of the original seaweed (poly)phenols catabolised
542 by the colonic bacteria but further studies would be required to confirm the identity of these
543 unknown compounds. Poor absorption of the high molecular weight phlorotannins in the upper
544 gastrointestinal tract following acute consumption of our seaweed (poly)phenolic extract as
545 reported (20) likely results in them reaching the colon and becoming subject to microbial
546 fermentation to lower molecular weight derivatives; recently confirmed by in vitro gut
547 modelling of SPE by colonic microbiota (15). The urinary metabolite profiles of seaweed
548 phenolics from the low and high excretors clearly indicated a high inter-individual variation in
549 metabolism similar to that observed in terrestrial plant phenolics, and no single common
550 metabolite or pattern could be detected following seaweed consumption. It is possible that
551 inter-individual variation in gut microbiota underlie these metabolic changes and were
552 responsible for the observed differences. That said, the study of bioavailability of seaweed

polyphenolics remains challenging due to the high range of molecular weight compounds present, and their characterisation is complicated further by the lack of commercially available standards. Other limitations of the present study include a relatively short-term intervention, results cannot therefore be extrapolated to long-term chronic consumption. Participants in this study may not be representative of the general overweight population of Northern Ireland as they were mostly recruited from the University of Ulster staff and local residents. While lymphocyte DNA damage (spontaneous DNA SBs) is considered appropriate for the substantiation of EFSA health claims in the context of protection against generic DNA damage, a lesion specific enzyme such as (ENDO III) would have been required for a claim related to oxidative DNA damage (51). Finally, the study population number may have been too small to yield significant results in intercellular cytokines. These factors should be considered for the design of future studies in this area.

To conclude, to the best of our knowledge, this work represents the first comprehensive study represents the first comprehensive study involving 78 human participants investigating the bioavailability of seaweed (poly)phenolics. Consumption of SPE, decreased DNA damage albeit to a modest extent in obese individuals only; with no clear effects on clinical markers of inflammation. While untargeted analysis identified novel urinary biomarkers of seaweed consumption and highlighted a high degree of inter-person variation in the metabolism of seaweed phenolics. Future studies that address the ingestion of seaweed phenolics will need to consider and adjust for these parameters, including the importance of establishing an individual's capacity for metabolising (poly)phenol (52-54).

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Table 1. Key components of phlorotannin rich basic seaweed extract, High Molecular Weight (HMW) seaweed extract fraction, and blend used for intervention capsules (SPE).

Extract component	**Basic seaweed extract** mg/175mg of extract	**HMW seaweed extract fraction** mg/50mg of extract	**Blend (SPE capsule)** mg/400mg capsule
Phlorotannin	61.25	46.05	107.3
Iodine	0.48	0.02	0.5
Maltodextrin[1]	0	0	175
Minerals	39.38	13	52.38
Fucoxanthin	0	36.5	36.5
Laminarin as glucose	10.68	1.75	12.43
Fucoidan as fucose	0	<0.001	<0.001
Mannitol	29.23	5.9	35.13
Inorganic arsenic	<0.001	<0.001	<0.001
Cadmium (LD 0.15mg/kg)	<LD	<LD	<LD
Mercury (LD 0.016mg/kg)	<LD	<LD	<LD
Lead (LD 1.1mg/kg)	<LD	<LD	<0.001
Tin (LD 1.7mg/kg)	<LD	<LD	<LD

[1]maltodextrin was added to the capsule formulation as an excipient.

Table 2. Baseline characteristics of the study population (n=80).

Variable	**Placebo treatment phase (n=80)**	**SPE treatment phase (n=80)**
Age (y)	42.8 ± 7.2	42.9 ± 7.1
Gender (M/W)	20/20	19/21
Height (m)	1.71 ± 0.08	1.72 ± 0.10
Weight (kg)	88.9 ± 14.1	89.1 ± 17.3
BMI (kg/m^2)	30.3 ± 3.5	30.0 ± 4.4

No significant differences P>0.05, Paired T Test.

Table 3. Habitual nutrient intake of subjects during intervention study

Variable intake	**Placebo treatment phase (n=77)[1]**	**SPE treatment phase (n=77)**	**% Change[2]**	**P value[3]**
Energy (kcal/d)	1949 ± 590[4]	2057 ± 684	5	0.110
Protein (g/d)	79.4 ± 28.6	79.9 ± 25.3	0.5	0.782
Carbohydrate (g/d)	220.6 ± 72.1	231.3 ± 84.2	5	0.278
Total fat (g/d)	78.6 ± 26.5	85.2 ± 34.8	8	0.058
Saturated fat (g/d)	29.0 ± 10.2	30.7 ± 15.3	6	0.417
Monounsaturated fat (g/d)	24.4 ± 9.3	26.8 ± 12.4	9	0.112
Polyunsaturated fat (g/d)	12.1 ± 5.4	13.5 ± 6.4	10	0.059
Fibre (g/d)[5]	12.9 ± 6.2	13.3 ± 4.8	3	0.259
Vitamin C (mg/d)	71.8 ± 50.5	69.4 ± 47.1	-3	0.909
Vitamin E (mg/d)	7.17 ± 3.7	7.67 ± 4.0	7	0.279
Folate (µg/d)	223.9 ± 98.3	228.2 ± 85.5	2	0.370
Carotene (µg/d)	2821 ± 1900	2553 ± 1706	-11	0.185

[1]S66 did not complete a food diary in either phase. [2] Calculated from SPE phase – Placebo phase. [3] Mean treatment group values were not significantly different between phases, P>0.05 (Wilcoxin signed rank test). [4] Mean ± SD. [5]Calculated using the Englyst method.

Table 4. Effects of seaweed polyphenol extract on lymphocyte DNA damage, CRP, blood lipids and F_2 isoprostanes

	Total (n=78)				Overweight (n=42)				Obese (n=36)			
Blood marker	**Average baseline value**	**Placebo treatment effect**	**SPE treatment effect**	**P value**	**Average baseline value**	**Placebo treatment effect**	**SPE treatment effect**	**P value**	**Average baseline value**	**Placebo treatment effect**	**SPE treatment effect**	**P value**
DNA damage – basal (% Tail)	6.72 ± 2.48	0.74 ± 2.86	-0.41 ± 3.13	0.350	6.59 ± 2.80	0.32 ± 2.14	0.57 ± 3.24	0.129	6.91 ± 2.00	1.81 ± 4.50	0.15 ± 2.93	**0.044**
DNA damage - H_2O_2 (%Tail)	34.2 ± 7.00	-1.56 ± 6.60	-2.03 ± 6.40	0.390	35.2 ± 7.11	0.13 ± 2.19	0.76 ± 1.66	0.062	32.8 ± 6.69	1.26 ± 2.09	0.54 ± 1.54	0.111
CRP (mg/ml)	2.67 ± 3.9	0.01 ± 3.3	-0.83 ± 4.9	0.429	2.59 ± 4.69	0.00 ± 3.40	-1.32 ± 6.22	0.348	2.80 ± 2.56	-1.81 ± 11.82	0.72 ± 5.15	0.258
Cholesterol (mmol/l)	5.20 ± 0.77	-0.06 ± 0.57	-0.10 ± 0.57	0.256	5.18 ± 0.82	0.01 ± 0.55	-0.53 ± 0.54	0.201	5.24 ± 0.73	-0.13 ± 0.58	-0.15 ± 0.60	0.419
Triglycerides (mmol/l)	1.51 ± 0.94	0.01 ± 0.82	0.04 ± 0.96	0.385	1.34 ± 0.72	0.07 ± 0.79	-0.04 ± 0.48	0.278	1.75 ± 1.15	-0.06 ± 0.85	-0.02 ± 1.34	0.497
HDL (mmol/l)	1.37 ± 0.32	-0.01 ± 0.15	0.03 ± 0.15	0.187	1.46 ± 0.31	0.01 ± 0.16	-0.03 ± 0.15	0.150	1.25 ± 0.32	-0.04 ± 0.11	-0.04 ± 0.14	0.446
LDL (mmol/l)	3.16 ± 0.1	-0.08 ± 0.5	-0.06 ± 0.50	0.478	3.13 ± 0.69	-0.07 ± 0.57	0.01 ± 0.45	0.383	3.20 ± 0.67	-0.09 ± 0.56	-0.13 ± 0.56	0.412
F_2Isoprostanes (pg/ml)	393 ± 220	-10 ± 182	-6 ± 138	0.374	342 ± 128	28 ± 152	-29 ± 108	0.130	439 ± 272	-44 ± 204	14 ± 158	0.283

Data is presented as treatment affects, calculated based on individual differences between pre- and post- values for both control and treatment phases for each subject. Paired T-tests were then carried out on the difference scores (post-treatment value *minus* pre-treatment values) between treatment (Seaweed phenolic extract capsule) and placebo control phase (maltodextrin). Significance level was set at $P<0.05$ (one-tailed T test).

Table 5. Phlorotannins metabolites tentatively identified in human urine samples from Group 1 (70 subjects) and Group 2 (8 subjects) after seaweed capsule consumption.

ID	VIP value	Rt (min)	Experimental Molecular Weight	Predicted Formula	Metabolite putative identification	Ratio[1]
Group 1 (72 subjects)						
652	2.06	25.6	205.9881	C6H6O6S	Pyrogallol/phloroglucinol sulfate	1.5
800	2.28	25.8	205.9881	C6H6O6S	Pyrogallol/phloroglucinol sulfate	1.2
1472	3.86	24.9	205.9881	C6H6O6S	Pyrogallol/phloroglucinol sulfate	1.1
1352	4.26	25.6	486.1727	C26H30O7S	Hydroxytrifuhaol A-glucuronide	2.0
1453	3.83	25.4	486.1727	C26H30O7S	Hydroxytrifuhaol A-glucuronide	1.9
1458	4.23	25.3	486.1727	C26H30O7S	Hydroxytrifuhaol A-glucuronide	2.2
1483	4.39	25.3	486.1727	C22H30O12	Hydroxytrifuhaol A-glucuronide	2.2
1917	2.11	31.4	544.2881	C27H44O11	Dioxinodehydroeckol glucuronide	1.1
Group 2 (8 subjects)						
293	2.08	9.5	327.0951	C12H8O9S	C-O-C dimer of phloroglucinol-sulfate	1.2
702	2.20	24.1	248.0315	C12H8O6	C-O-C dimer of phloroglucinol	1.2
853	2.08	27.8	797.3186	C45H49O13	Fucophloroethol glucuronide	1.1
1293	2.28	34.2	330.1675	C12H10O9S	Diphlorethol sulfate	1.9
1356	2.31	34.1	330.1675	C12H10O9S	Diphlorethol sulfate	1.7
1633	2.07	35.1	544.2152	C27H44O11	Dioxinodehydroeckol glucuronide	1.2

ID, identification number. VIP (variable influence in projection) is a variable that summarizes the importance of X variables to the OPLS-DA model. Variables with values > 2 were the most influential in the model. All predicted formula derived with < 5 ppm mass accuracy data.

[1]Ratio: seaweed capsule consumption/placebo consumption.

FIGURE 1. CONSORT DIAGRAM. Progress of participants through the intervention study.

FIGURE 2. Comparison of the effects of SPE supplementation phase (■) with that of a placebo phase (□) on total oxidative capacity (TOC) in the total study population (n=78), in overweight (n=42), and in obese (n=36). All values were measured in duplicate expressed as mean ± SD. TOC is represented as the mean of the individual difference values (after – before supplementation) in the SPE and placebo treatment phases.

FIGURE 3. Comparison of the effects of SPE supplementation phase (■) with that of a placebo phase (□) on cytokine levels in the total study population (n=78), in overweight (n=42), and in obese (n=36). All values were measured in duplicate expressed as mean ± SD. Cytokines were measured as the mean of the individual difference value (after – before supplementation) in the SPE and placebo treatment phases. Change in mean treatment group values were not significantly different between treatment phases, $P>0.05$ (Paired T Test; one-tailed test). M.F.I.; Mean Fluorescence Intensity.

FIGURE 4. Principal Component Analysis (PCA) (A) and Hierachical Cluster Analysis Cluster (HCA) (B) of urinary profiles before (♦) and after (■) seaweed consumption by 80 participants. The HCA was calculated based on Eucledian distances and the Ward hierachical agglomerative method. The PC explained 23.14% of the total variance (PC-1 14.8% and PC-2 8.34%).

FIGURE 5 A) OPLS-DA scores and B) loadings of the urine samples belong to group of participants 1 (70 participants). C) OPLS-DA scores and D) loading of urine samples belong to group of participants 2 (8 participants) before (♦) and after (■) seaweed ingestion. (Circles shown in the graph represent a confidence of 95%). LV1: latent variable 1; LV 2: latent variable 2. The cut off VIP value selected to be 2. For VIP scores identification see **Table 5** and Supplemental **Table 1**.

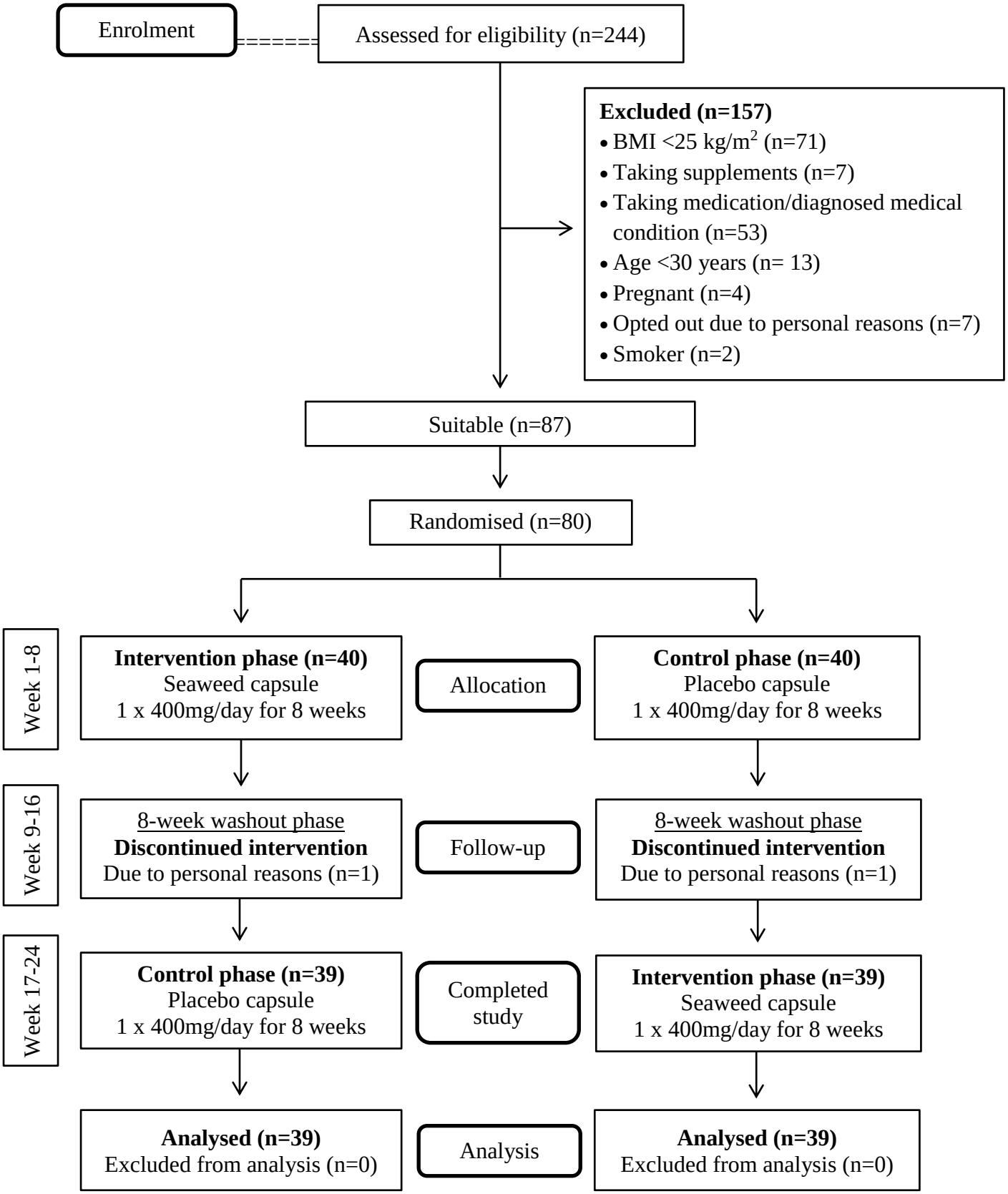
Enrolment
Assessed for eligibility (n=244)
Excluded (n=157)
• BMI <25 kg/m2 (n=71)
• Taking supplements (n=7)
• Taking medication/diagnosed medical condition (n=53)
• Age <30 years (n= 13)
• Pregnant (n=4)
• Opted out due to personal reasons (n=7)
• Smoker (n=2)
Suitable (n=87)
Randomised (n=80)
Week 1-8
Intervention phase (n=40)
Seaweed capsule
1 x 400mg/day for 8 weeks
Allocation
Control phase (n=40)
Placebo capsule
1 x 400mg/day for 8 weeks
Week 9-16
8-week washout phase
Discontinued intervention
Due to personal reasons (n=1)
Follow-up
8-week washout phase
Discontinued intervention
Due to personal reasons (n=1)
Week 17-24
Control phase (n=39)
Placebo capsule
1 x 400mg/day for 8 weeks
Completed study
Intervention phase (n=39)
Seaweed capsule
1 x 400mg/day for 8 weeks
Analysed (n=39)
Excluded from analysis (n=0)
Analysis
Analysed (n=39)
Excluded from analysis (n=0)

FIGURE 1

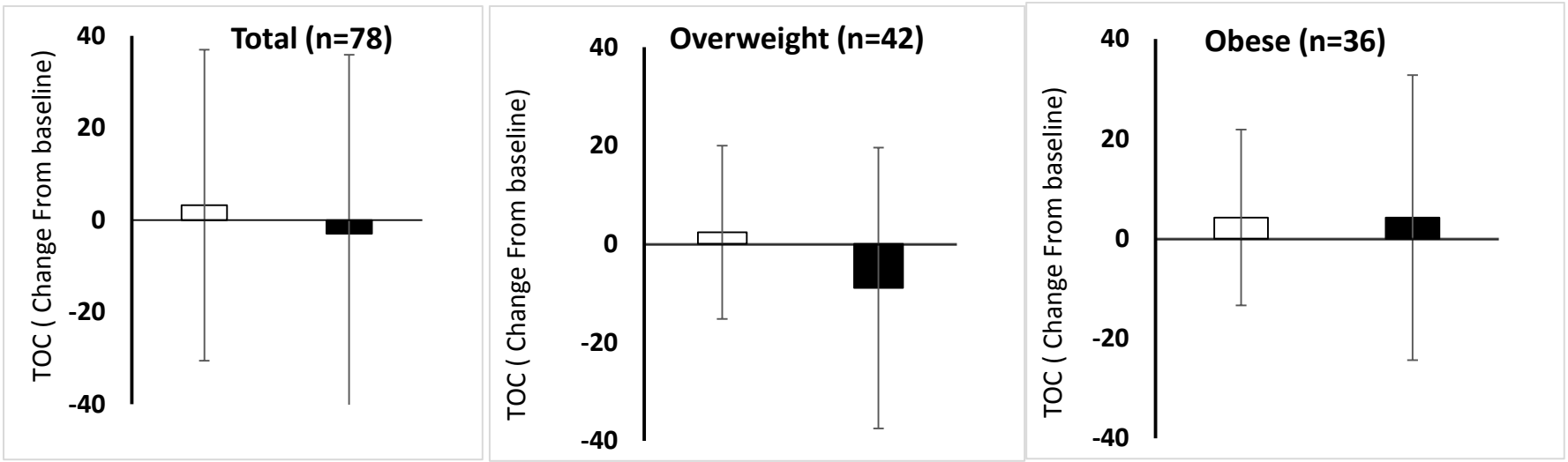
Total (n=78)
Overweight (n=42)
Obese (n=36)
TOC (Change From baseline)
40
20
0
-20
-40

FIGURE 2.

FIGURE 3.

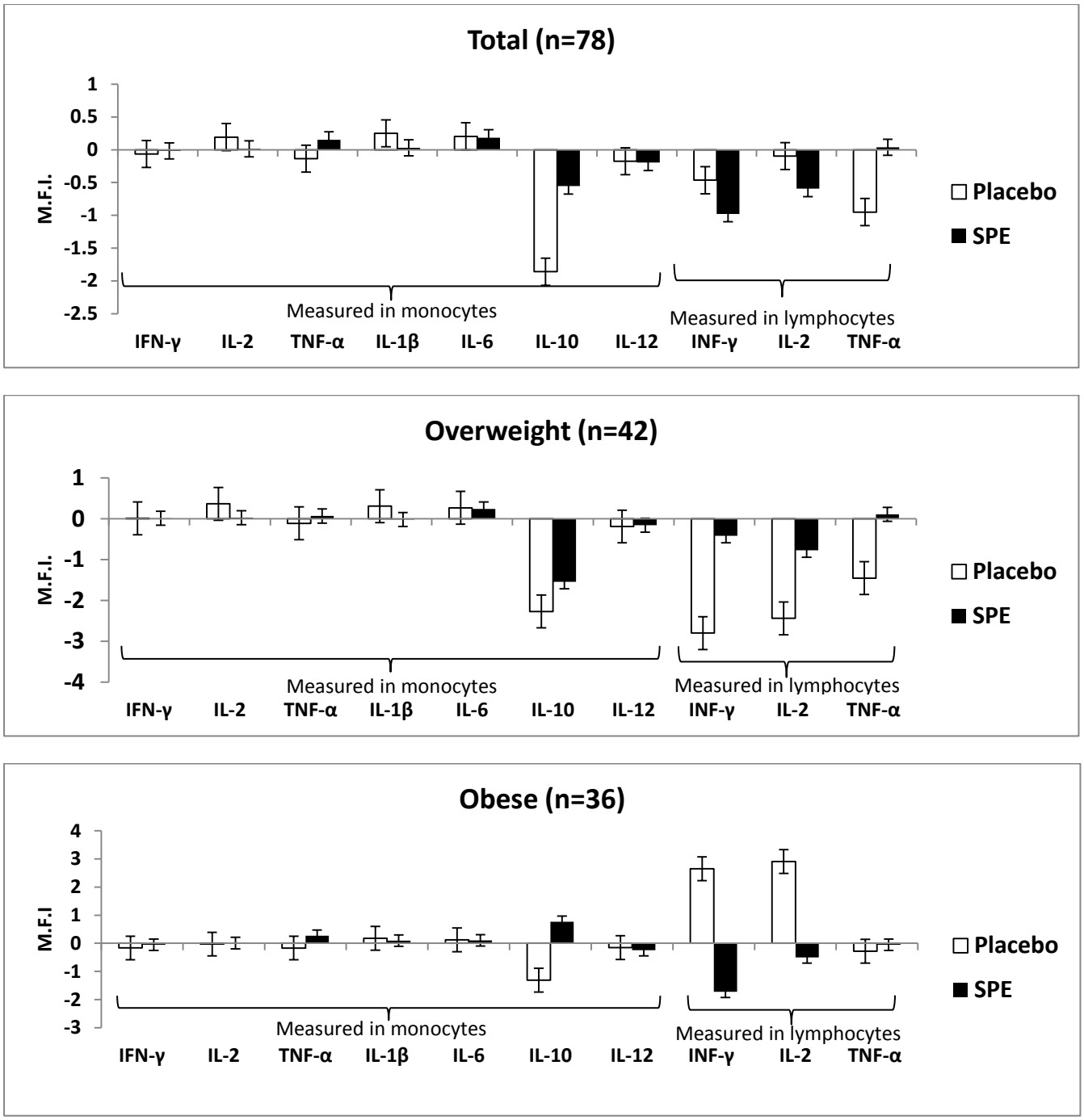

FIGURE 3.

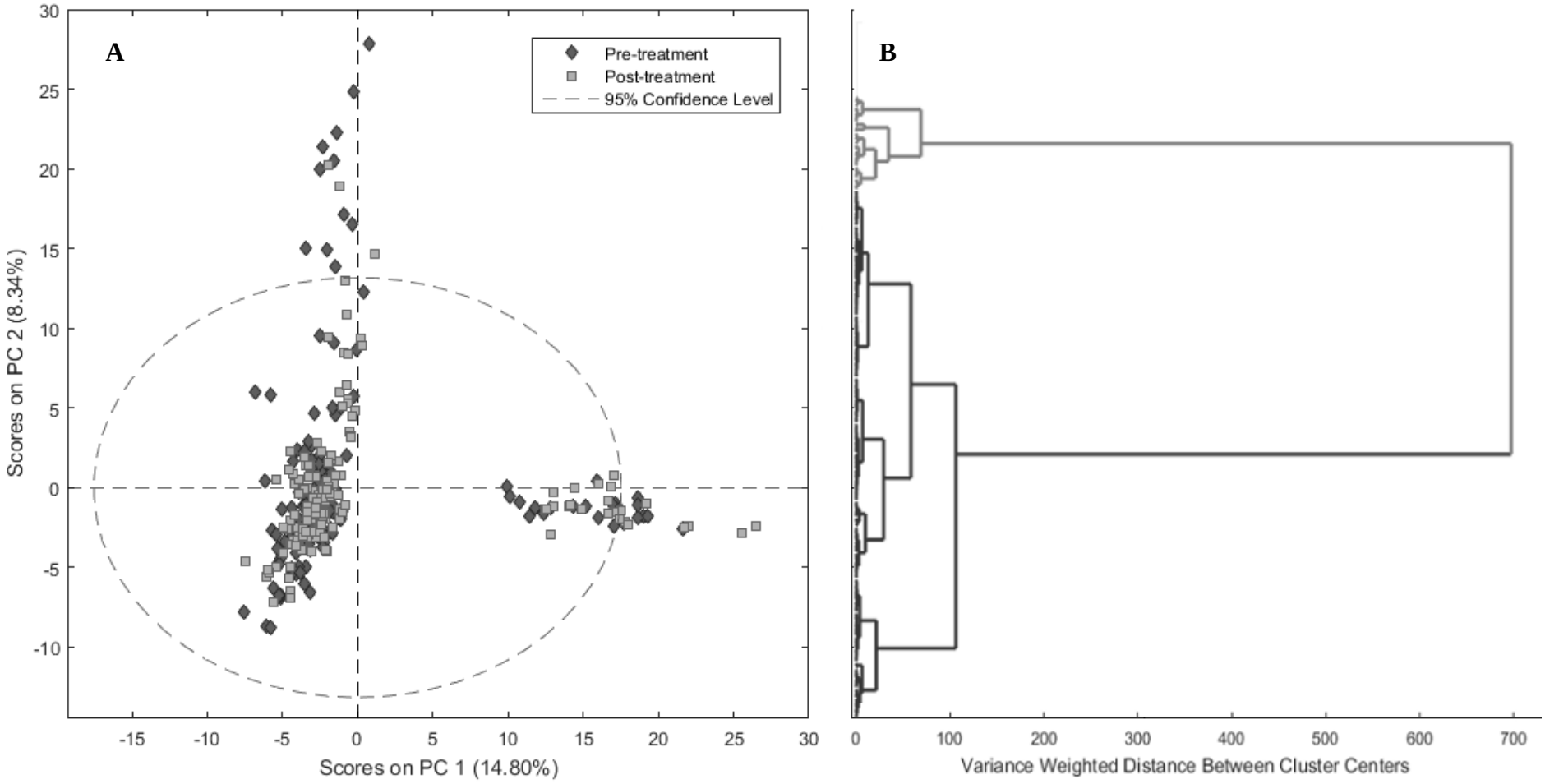
A
Pre-treatment
Post-treatment
95% Confidence Level
Scores on PC 2 (8.34%)
Scores on PC 1 (14.80%)
B
Variance Weighted Distance Between Cluster Centers

FIGURE 4.

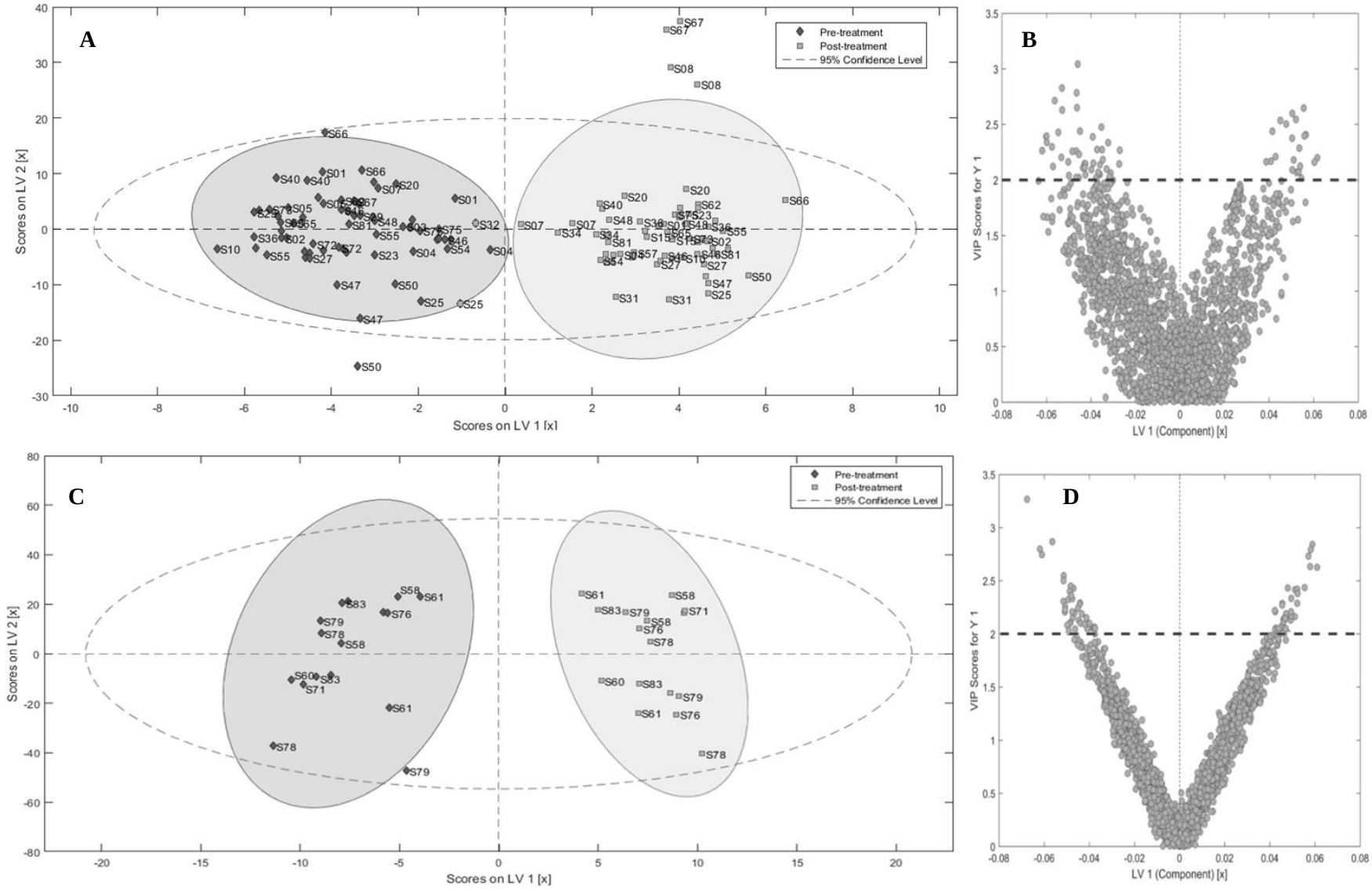
A
Pre-treatment
Post-treatment
95% Confidence Level
Scores on LV 2 [x]
Scores on LV 1 [x]
B
VIP Scores for Y 1
LV 1 (Component) [x]
C
Pre-treatment
Post-treatment
95% Confidence Level
Scores on LV 2 [x]
Scores on LV 1 [x]
D
VIP Scores for Y 1
LV 1 (Component) [x]

FIGURE 5